The tyrosine phosphatase SHP-1 as a new player that dampens murine Th17 development

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Abstract

Th17 cells represent a subset of CD4+ T helper cells that secrete the pro-inflammatory cytokine IL-17. Th17 cells have been ascribed both a beneficial role in promoting clearance of pathogenic fungi and bacteria, and a pathogenic role in autoimmune diseases. Here, we identify the tyrosine phosphatase SHP-1 as a critical regulator of Th17 development, using three complementary approaches. Impaired SHP-1 activity through genetic deletion of SHP-1, transgenic expression of an inducible dominant negative SHP-1, or pharmacological inhibition of SHP-1 strongly promotes the development of Th17. Ex vivo Th17 skewing assays demonstrate that genetic or pharmacological disruption of SHP-1 activity in T cells results in a hyper-response to stimulation via IL-6 and IL-21, two cytokines that promote Th17 development. Mechanistically, we find that SHP-1 decreases the overall cytokine-induced phosphorylation of STAT3 in primary CD4+ T cells. These data identify SHP-1 as a key modifier of IL-6 and IL-21-driven Th17 development via regulation of STAT3 signaling, and suggest SHP-1 as a potential new therapeutic target for manipulating Th17 differentiation in vivo.
Introduction

Th17 cells are a subset of CD4+ T helper cells defined by their ability to secrete IL-17A and IL-17F. IL-17 is an inflammatory cytokine important in mediating host defense against bacterial and fungal pathogens. Under physiological conditions, Th17 cells are found in the intestinal lamina propria and Peyer’s patches, where they are regulated by the local cytokine milieu and support responses against pathogenic bacteria and fungi. However, unregulated Th17 development and IL-17 production have been shown to contribute to the development of allergic and autoimmune diseases. Recently, Th17 cells have also been linked to cancer, but their involvement towards cancer ablation or progression varies widely depending on the type of cancer. Therefore, characterizing the intracellular signaling within CD4+ T cells that modifies Th17 development will have important clinical implications for a broad range of diseases. To date, few studies have addressed how modifying early signaling events in CD4+ T cells affects Th17 differentiation.

Stimulation of naïve T cells with either IL-6 + TGF-β or IL-21 + TGF-β leads to the activation and induction of several key transcription factors essential for Th17 differentiation, including STAT3, RORγt, and RORα. The signaling cascade via the IL-6 receptor leads to the downstream activation of Jak kinases, and in turn Jak-mediated phosphorylation of STAT3 proteins. This leads to homo-dimerization and translocation of STAT3 into the nucleus, where STAT3 directly binds to the il17a promoter, and is required for the induction of RORγt. Consistent with this, STAT3-/- mice completely lack Th17 cells and are resistant to experimental autoimmune encephalitis (EAE). To date, a network of transcription factors has been linked to Th17 differentiation,
yet modifiers of the signaling cascade from cytokine stimulation to transcription, and in turn Th17 development, are not well understood 20.

The Src homology region 2 domain-containing tyrosine phosphatase-1 (SHP-1) is a cytoplasmic protein tyrosine phosphatase expressed in all hematopoietic cell lineages. Motheaten (me/me) mice are homozygous for a mutation that abrogates SHP-1 protein expression, and display hematopoietic abnormalities resulting in death approximately 2-3 weeks after birth. SHP-1 is a negative regulator of signaling via cytokines, chemokines, growth factors and antigens 21. Specifically, SHP-1-deficient T cells display increased responses to TCR stimulation, and concomitant hyper-proliferation in ex vivo cultures 22. However, the role of SHP-1 during Th17 cell differentiation is not known. Here, using mice in the motheaten background, as well as a new tissue-specific transgenic mouse line expressing a dominant negative mutant of SHP-1 in T cells, we demonstrate that SHP-1 naturally dampens Th17 cell development in vivo. SHP-1-deficient mice have increased percentages of Th17 cells in their Peyer's patches and intestinal lamina propria, and T cells with decreased SHP-1 activity hyper-respond to IL-6 or IL-21 stimulation, in turn generating higher numbers of Th17 cells. As an independent non-genetic approach, we used SSG, a small molecule inhibitor of SHP-1 activity 23,24 that is currently tested in clinical trials as treatment option of patients with advanced solid tumors 25-27. SSG-mediated inhibition of SHP-1 again demonstrated the regulatory role of SHP-1 in Th17 differentiation. Mechanistically, SHP-1 decreases the tyrosine phosphorylation of STAT3 after IL-6 or IL-21 stimulation, thereby directly dampening a transcription factor critical for Th17 development. Collectively, these data identify SHP-1 as a new player that naturally regulates Th17 cell differentiation in vivo.
Materials and methods

Mice

*me/+* (C57BL/6) mice were bred to generate +/+, *me/+ and *me/me* mice. *me/+* DO11.10 TCR-Tg*+ (BALB/c) mice were bred to generate +/+ DO11.10 TCR-Tg*, *me/+ DO11.10 TCR-Tg*, and *me/me DO11.10 TCR-Tg*. Genotyping for all mice was performed as previously described 23. C57BL/6 and *rag1-/-* (C57BL/6) were obtained from Jackson laboratories (Bar Harbor, ME). CD4-Cre (C57BL/6) mice were obtained from Taconic (Hudson, NY) 28. For all experiments including *me/me* mice, 15-19 day old mice were used. For all other studies, 4-6 week old mice were used.

The DN-SHP-1 construct (SHP-1-D419A) was subcloned into the modified pLITMUS28 plasmid, in which the EF-1α promoter and DN-SHP-1 cDNA were separated by a transcription-translation STOP cassette with flanking loxP sites (Fig. 4A) 29. DN-SHP-1 mice were generated by the UVA Transgenic Core Facility and bred onto the C57BL/6 background for more than 12 generations. DN-SHP-1 Tg*+* C57BL/6 mice were crossed with CD4-Cre (C57BL/6) expressing mice to drive T cell-specific expression of DN-SHP-1. Genotyping and confirmation of stop cassette deletion (Fig. S3A) was performed using the following primers from Integrated DNA Technologies (Coralville, Iowa); 5’ loxP 3040: 5’-GGG GCT CTA GTG AAC CTC TCC G-3’; 3’ loxP 3506: 5’-GAT AGG TGG CAA GTG GTA TTC CG-3’; 3’ SHP1 674: 5’-CAC CCT CGT GGC ATA GTA CGG C-3’.

All mice were bred and maintained in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) at the University of Virginia. All experiments involving mice were conducted with the approval of the IACUC.
Isolation of primary cells from Peyer’s patches and intestinal lamina propria.

Intestines were isolated from mice and placed in complete media (RPMI-1640 from Invitrogen, Carlsbad, CA, supplemented with 10% FBS, 5x10^{-5} M 2-β-mercaptoethanol (2-ME), 2 mM L-glutamine, 10 mM HEPES, 20 mM sodium bicarbonate, and antibiotics. Peyer’s patches were isolated manually from the intestine surface with forceps, and cells were dispersed by straining through a 40 mm cell strainer. The intestines were cut longitudinally and washed in Phosphate Buffered Saline (PBS) followed by 20 min incubation at 37°C in Hank’s Buffered Salt Solution (HBSS) supplemented with 5 mM EDTA. The intestines were washed again in PBS, cut into 1.5 cm pieces, and digested in 50 ml of digestion solution (RPMI-1640 supplemented with 10% FBS, 5 mM 2-ME, 1 mg/ml collagenase type II (Worthington Biochemical, Lakewood, NJ), 1 mg/ml Dispase (Invitrogen, Carlsbad, CA), and 40 μg/ml DNase I (Roche, Branford, Connecticut) for 1 hour at 37°C. Following digestion, intestinal pieces were strained through a 40mm cell strainer to collect released lamina propria cells which were then stimulated and stained as described below.

Cell Staining and Flow Cytometry

For intracellular cytokine staining, cells were seeded at 2x10^6 cells/ml in complete media and incubated for 4-5 hours with 60 ng/ml Phorbol-12-myristate-13 acetate (PMA) (Calbiochem, San Diego, CA), 750 ng/ml Mixed Calcium Magnesium Salt (Calbiochem, San Diego, CA), and 1.0 ml/ml BD GolgiStop (BD Biosciences, San Jose, CA). Cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) and stained with the appropriate antibodies. Staining antibodies: CD4-FITC, CD8-FITC, CD4-PE, IL-17-PE, IL-17A-PE, IgG1-PE, IFNγ-APC, IgG1-APC, CD4-Alexa647, CD4-PE, CD4-Percp, TCRβ chain-APC, CD45RB-Fitc, CD45RB-PE, CD25-APC (BD Bioscience,
San Jose, CA). IL-17F-Alexa647, IgG2-Alexa647, IL-6Rα-PE, IL-21R-PE, IL-21-PE (eBioscience, San Diego, CA). Stained cells were collected on the FACSCalibur instrument using the CellQuest software (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR). Analyses were conducted on live cells (>95%) as defined by forward and side-angle scatter. Gates were set based on isotype-matched controls.

**IL-6 ELISA**

Orbital eye bleeds were performed to collect sera. Concentrations of IL-6 in sera were measured using the BD OptEIA mouse IL-6 ELISA Set with BD OptEIA buffers (BD Bioscience, San Jose, CA).

**Colitis transfer model**

Naïve splenic CD4+ T cells were isolated (>98%) from +/- or mel/+ C57BL/6 mice via negative selection as described below, and stained with DAPI (Sigma-Aldrich, St Louis, MO) CD4-Fitc, CD25-APC, CD45RB-PE (BD Bioscience, San Jose, CA). CD4+CD25-CD45RBhi T cells were sorted in the UVA Flow Cytometry Core facility to ≥ 95% purity. 5x10^5 CD4+CD25-CD45RBhi T cells were i.p. injected into rag1/- mice. Mice were observed daily and weighed weekly. Mice developed clinical signs of colitis by 7-8 weeks post transfer and were euthanized for analysis. Colon segments were fixed in 10% formalin, embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin (H&E) for pathological scoring. Colitis severity was scored semi-quantitatively from 0 - 4 in a blinded fashion as follows: Grade 1 indicates focal monocytic inflammation at the base of the glands. Grades 2 - 3 indicate incremental inflammation and epithelial changes with severity above grade 1, but below grade 4. Grade 4 indicates a loss of all
goblet cells, hyperplastic and dysplastic glandular epithelium and massive inflammation through all bowel layers with giant cells and crypt abscesses.

**Th17 culture conditions**

CD4⁺ T cell purification: Splenocytes were dispersed followed by red blood cell lysis using BD Pharm lyse buffer (BD Pharmingen, San Jose, CA). CD4⁺ T cells were isolated from the spleen by negative selection using the CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol.

Th17 culture: 1.5x10⁵ CD4⁺ T cell together with 1.5x10⁵ T cell-depleted splenocytes serving as antigen presenting cells (APC, irradiated at 2000 RAD) were seeded per well into 24 well plates in 500ul complete RPMI 1640 media supplemented with: 1.25 µg/ml anti-CD3 Ab (145-2c11; Cedarland Laboratories, Burlington, NC), or 5 µg/ml OVA 323-339(ISQAVHAAHAEINEAGR) peptide (Bimolecular Research Facility, UVA), 1 µg/ml anti-CD28 Ab (Southern Biotech, Birmingham, AL), 5 ng/ml TGF-β (R&D Systems, Minneapolis, MN and eBioscience, San Diego, CA), 40 ng/ml IL-23, 5 µg/ml anti-IL-4 Ab, 5 µg/ml anti-IFNγ Ab, and IL-6 (0 - 75 ng/ml), or IL-21 (0 - 200 ng/ml) purchased from eBioscience (San Diego, CA). Cells were harvested 3-5 days later, and re-stimulated as described.

SSG treatment during Th17 culture: Where indicated, SSG (10 µg/ml; Calbiochem, San Diego, CA) was added to the culture. SSG used at 10 µg/ml has been determined to inhibit 99% of SHP-1 activity with no detectable effect on other phosphatases ²³,²⁴.

EC50 determination for IL-6 and II-21 dose responses: To determine the EC50 of IL-6 and IL-21 for optimal TH17 differentiation, the maximal percentage of differentiated Th17 cells per experiment was set to 100%, and all other percentages were calculated in
relation to this highest Th17 population. These values were then plotted against the IL-6 or IL-21 concentrations to determine the EC50 values.

**Immunoblotting**

Splenic CD4$^+$ T cells were isolated as described above. 0.7x10$^6$ CD4$^+$ T cells were stimulated with 50 ng/ml IL-6 or 50 ng/ml IL-21 at 37$^\circ$ C for 0 - 60 min. before lysis in SDS buffer (2% SDS, 50mM Tris pH 7.6, 10% glycerol, 5% 2-ME and protease inhibitors as described previously). Lysates were separated via 10% SDS-PAGE followed by transfer and immunoblotting using the following antibodies: anti-pSTAT3-Tyr705 (clone D3A7, 1:500 dilution), anti-STAT3 (clone 124H6, 1:500 dilution) (Cell Signaling, Danvers, MA), and anti-β-Actin (clone AC-15 Peroxidase-labeled, 1:40,000 dilution, Sigma, St Louis, MO). Relative protein levels were calculated based on densitometry measurements of immunoblots at linear range using the ImageJ program (NIH, Bethesda, MD).

**Quantitative RT-PCR**

Splenic CD4$^+$ T cells were purified as described and total RNA was extracted using the RNeasy mini kit followed by DNase digestion (RNase Free DNase Set; Qiagen, Valencia, CA). cDNA was generated with the Superscript III First strand kit (Invitrogen, Carlsbad, CA). qRT-PCRs for STAT3 and HPRT1 were performed using the TaqMan Fast Universal PCR Master mix and commercially available Taqman Gene expression assays for STAT3 and HPRT1 (Applied Biosystems, Carlsbad, CA). Using HPRT1 expression for normalization, relative STAT3 expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

**Statistical analysis**

$p$ values were calculated using the unpaired Student's t-test. $p$ values of less than 0.05 were considered significant.
Results

\textit{SHP-1 limits natural intestinal Th17 development.}

Th17 cells are naturally present in the Peyer’s patches and the lamina propria of the small intestines in mice where they are thought to be induced by local cytokine responses that occur in response to bacteria and fungi. We first asked whether SHP-1 affected intestinal Th17 development \textit{in vivo} using the \textit{motheaten} DO11.10 mouse model \textsuperscript{23,30}. DO11.10 mice express a transgenic (Tg) T cell receptor (TCR) derived from the DO11.10 T cell hybridoma line, which specifically recognizes chicken OVA peptide, aa 323-339 in the context of I-A\textsuperscript{d} \textsuperscript{30}. SHP-1-deficient \textit{me/me} mice and \textit{me/+} BALB/c DO11.10 mice had higher percentages of CD4\textsuperscript{+} IL-17A/F producing cells in their Peyer’s patches and intestinal lamina propria, compared to +/+ littermates (Fig. 1A top, C-D). This was also seen in a non-TCR transgenic background, \textit{me/me} and \textit{me/+} C57BL/6 mice also displayed increased levels of intestinal Th17 cells in their Peyer’s patches (Fig. 1A bottom). Since Th17 cells have been demonstrated to contribute to colitis, we examined the intestines for pathological signs of disease or damage. However basally, the intestines of \textit{me/+} and \textit{me/me} mice showed no signs of intestinal damage or inflammation despite having higher percentages of Th17 cells (Fig. 1B).

We then asked whether the Th17 representation in other lymphoid tissues was altered in the absence of SHP-1. Comparable Th17 cell populations were seen in the inguinal and mesenteric lymph nodes of \textit{me/+} mice and +/+ mice (Fig. 1E), but a small increase in the population of Th17 cells was observed in the spleens of \textit{me/me} BALB/c/DO11.10 and \textit{me/me} C57BL/6 mice (Fig. 1E). This suggested that the effect of SHP-1 deficiency on Th17 development is not widespread and largely limited to the physiological localization of Th17 cells.
Since IL-6 is a critical cytokine for Th17 differentiation, we next examined IL-6 levels in +/-, me/+ and me/me mice. While comparable low levels of IL-6 were found in the sera of wild type (+/+) and heterozygous me/+ mice, significantly higher levels of IL-6 were observed in the sera of homozygous me/me mice (Fig. S1 A-B). Upon analyzing many homozygous me/me mice, we consistently found a correlation between higher sera levels of IL-6 and an increase in splenic Th17 cells. We also observed that me/me mice in the C57BL/6 background had higher sera levels of IL-6 compared to me/me mice on the BALB/c background, which again correlated with larger percentages of splenic Th17 cells.

Since increased IL-6 sera levels in me/me mice appear to contribute to higher percentages of splenic Th17 cells and to avoid the potentially complicating effects that continuous exposure to IL-6 creates, we focused on comparing heterozygous me/+ with +/- mice for all of the following studies addressing Th17 differentiation. Although +/- and me/+ mice have comparable sera IL-6 levels and comparable populations of Th17 cells in their spleens, me/+ mice still have an increased Th17 cell population in their lamina propria and Peyer’s patches, suggesting that SHP-1 directly regulates Th17 differentiation.

T cells from SHP-1 deficient mice induce colitis.

Th17 cells have been causally associated in the pathogenesis of colitis \(^1,^{12,31}\). Since me/+ and me/me mice showed no pathological signs of intestinal disease at steady state, we asked whether SHP-1-deficient cells are capable of inducing colitis. In the colitis transfer model, adoptive transfer of CD4\(^+\)CD25\(^-\)CD45RB\(^{\text{high}}\) T cells into rag1\(^{-/-}\) recipients induces colitis within 7-8 weeks \(^{31,32}\), and the Th17 cell-associated cytokines impact colitis development and progression. Therefore, we transferred CD4\(^+\)CD25\(^-\)CD45RB\(^{\text{high}}\) T cells purified from +/- and me/+ C57BL/6 mice into rag1\(^{-/-}\) recipients, and assessed colitis severity and Th17 cell populations. Over a period of 7-8 weeks, the recipients of me/+ as well as +/- cells developed diarrhea with associated weight loss. Histological examination
of the intestines confirmed that both +/+ and me/+ diseased mice exhibited colon thickening, comparable degree of monocytic and neutrophilic infiltration, a reduction in goblet cells, and moderate epithelial hyperplasia (Fig. 2 A-B). Furthermore, we observed comparable percentages and absolute numbers of the IL-17A/F producing cells in the Peyer’s patches and intestinal lamina propria in recipients of cells from SHP-1-deficient or control mice (Fig. 2 C-D). Thus, the Th17 cells derived from SHP-1-deficient mice are functional and capable of inducing pathogenesis in this model of colitis.

**SHP-1 negatively regulates IL-6 mediated signaling in a T cell intrinsic manner.**

We observed enhanced Th17 development in the intestines of me/+ mice compared to +/+ mice, suggesting the possibility that SHP-1 might regulate Th17 development, and that the absence of SHP-1 could then manifest as higher numbers of Th17 cells. Both IL-6 and IL-21 promote Th17 cell differentiation by inducing STAT3 activation, which in turn regulates RORγt and RORα activation. To address the possible link between SHP-1, IL-6 signaling and Th17 development, we performed Th17 lineage differentiation assays using an IL-6 dose response (while maintaining a constant optimal level of TGF-β, Fig. S1C). T cells derived from me/+ mice developed increased percentages and numbers of Th17 cells, especially at the lower range of IL-6 concentrations compared to +/+ T cells (Fig. 3 A-B, and Fig. S1D). Furthermore, T cells from me/+ mice reached their half maximal effective concentration (EC50) at 0.25 ng IL-6 compared 1.0 ng for +/+ T cells. This four-fold reduction in EC50 for me/+ cells suggests that SHP-1-deficient T cells are hyper-responsive to IL-6 stimulation. Both +/+ and me/+ T cells respond similarly at higher concentrations of IL-6, indicating that the regulatory effect of SHP-1 on IL-6 signaling and Th17 differentiation is most pronounced at suboptimal IL-6 concentrations.

We have previously demonstrated that SHP-1 negatively regulates TCR-mediated signaling. Therefore, to exclude TCR-mediated effects, we used optimal levels of TCR
stimulation where effects of SHP-1 on TCR-mediated signaling are not detectable. Consistently, we observed higher percentages of Th17 cells in cultures of me/+ T cells compared to +/+ T cells at all antigen concentrations tested (Fig. S1E). Finally, comparable numbers and percentages of IFNγ+ T cells were generated from +/+ and me/+ T cells suggesting a preferential influence of SHP-1 on Th17 differentiation (Fig. 3A, Fig. S1F).

Since T cells from me/+ mice are hyper-responsive to IL-6 stimulation, we asked whether SHP-1-deficient T cells expressed higher levels of IL-6R, which might have been advantageous. However, T cells isolated from SHP-1-deficient and +/+ mice express comparable expression levels of IL6Rα (Fig. 3C) suggesting that differences in intracellular signaling downstream of the IL-6R are responsible for the hyper-responsiveness of the SHP-1-deficient T cells.

In the context of the in vivo me/+ mouse studies, the phenotypes observed in me/+ mice could be complicated by secondary effects by other lineages since the me mutation is present in every hematopoietic cell lineage 21. Therefore, to test whether SHP-1 can influence Th17 differentiation in a T cell intrinsic manner, we generated transgenic mice that can conditionally express a dominant negative mutant of SHP-1 (DN-SHP-1). Due to the loxP-flanked STOP cassette in front of the coding sequence for DN-SHP-1, its expression is conditional and can be induced in a tissue specific manner (Fig. 4A, Fig. S3A). The DN-SHP-1 mutant contains an aspartic acid to alanine point mutation (D419A) in the WPD loop of the phosphatase, which renders it catalytically inactive and enables it to act as a dominant negative mutant 33. DN-SHP-1 transgenic mice were crossed with CD4-Cre transgenic mice, where the Cre expression is under the CD4 promoter and Cre-mediated deletion occurs at the late DN4 and DP stages 28. Thymic cellularity and CD4/CD8 profiles were comparable between DN-SHP-1 expressing and non-expressing
mice indicating that T cell-specific DN-SHP-1 expression does not affect overall thymic development (Fig. S3B). Next we assessed whether DN-SHP-1 expression was functional. The DN-SHP-1 expressing T cells hyper-respond to TCR stimulation at lower concentrations of OVA peptide, similar to me/+ mice, confirming that the transgenically expressed DN-SHP-1 protein can act in a dominant negative fashion (Fig. S3C). To test the effect of DN-SHP-1 on Th17 differentiation, T cells were purified and exposed to a range of IL-6 concentrations under Th17 skewing conditions. DN-SHP-1 expressing T cells were also hyper-responsive to IL-6 stimulation, manifested by increased generation of Th17 cells at sub-optimal IL-6 concentrations (Fig. 4 B-C). Furthermore, T cells from DN-SHP-1 expressing mice reach their EC_{50} at a ~2-3-fold lower IL-6 concentration than non-expressing T cells (1.0 ng/ml IL-6 vs. 2.5 ng/ml IL-6, Fig. 4C). To focus on the responsiveness to IL-6 in these assays, the T cells were cultured in the presence of optimal TGF-β and TCR stimulation. Collectively, based on two different in vivo models (the me mutation and transgenic expression of dominant negative SHP-1), these data suggest that SHP-1 negatively regulates IL-6 driven Th17 development in a T cell intrinsic manner.

We next asked whether disturbing SHP-1 function in the context of wild type T cells would also favor their development to Th17 lineage. For these studies, we took advantage of the drug Sodium Stibogluconate (SSG), which specifically inhibits SHP-1\(^24\). Purified CD4\(^+\) T cells from wild type C57BL/6 mice were cultured under Th17 promoting conditions in the presence or absence of SSG, and in a dose response with IL-6. T cells in the SSG-treated conditions were hyper-responsive to sub-optimal IL-6 leading to increased numbers of Th17 cells (Fig. 5A). SSG-treated T cells reached their EC_{50} at a ~4-fold lower concentration of IL-6 compared to untreated T cells (0.25 ng/ml IL-6 vs. 1.0 ng/ml IL-6). Both SSG-treated and untreated T cells responded similarly at higher
concentrations of IL-6, again supporting our finding that SHP-1 is a negative regulator of IL-6 signaling at suboptimal IL-6 concentrations and thereby influences Th17 differentiation. To confirm that under our conditions SSG is a specific inhibitor of SHP-1, we skewed T cells purified from +/+, me/+, and me/me mice towards the Th17 lineage. SSG treatment of +/+ and me/+ T cells, but not me/me T cells, causes a hyper-response to IL-6 stimulation as evidenced by the generation of increased percentages (Fig. 5B) and absolute numbers (data not shown) of Th17 cells when compared to untreated T cells. Thus, the observed effect of SSG on IL-6-driven Th17 differentiation appears to be directly due to changes in SHP-1 activity (Fig. 5B). Remarkably, the effect of SSG on Th17 differentiation essentially reproduced our data in the genetic models with me/+ and DN-SHP-1 T cells. Collectively, based on the three different approaches, SHP-1 negatively regulates IL-6-mediated Th17 differentiation in a T cell intrinsic manner.

**SHP-1 regulates IL-6 dependent STAT3 phosphorylation.**

We next sought to understand the molecular mechanism, by which SHP-1 might regulate IL-6 dependent Th17 differentiation. IL-6 stimulation has been shown to induce the activation of the Jak/STAT3 pathway, which in turn directly regulates RORγt and RORα activation and Th17 cell differentiation. To address whether SHP-1 is a negative regulator of Jak/STAT3 signaling, purified CD4⁺ T cells from +/+, me/+ or me/me mice were stimulated with IL-6 and examined for levels of induced STAT3 phosphorylation as an indicator of STAT3 activation. We made two key observations. First, SHP-1-deficient T cells had overall higher levels of phosphorylated STAT3 in response to IL-6 stimulation when compared to +/+ T cells, while the kinetics of STAT3 phosphorylation were unaffected by SHP-1 (Fig. 6 A-B). Second, surprisingly, we also observed higher levels of STAT3 protein in T cells from me/me mice and some of the me/+ mice (Fig. 6A). As noted earlier there is some heterogeneity among the me/+ mice,
and the subset of \textit{me}+/ mice that had STAT3 protein levels comparable to +/+ mice still had higher levels of phosphorylated STAT3 in response to IL-6 stimulation (Fig. 6C).

We also asked whether SHP-1 is affecting the generation of STAT3 protein at the transcriptional level. qRT-PCR for \textit{Stat3} message on T cells purified from +/+, \textit{me}+/ and \textit{me}/me mice found comparable levels of \textit{Stat3} mRNA between +/+, \textit{me}+/ and \textit{me}/me T cells (Fig. 6D). This indicates that SHP-1 does not affect STAT3 transcription, rather suggesting a regulatory mechanism at the protein level. Thus, SHP-1 appears to negatively regulate STAT3 signaling at two levels, by affecting STAT3 phosphorylation (as seen in the \textit{me}+/ mice) and by altering STAT3 protein levels. Collectively, our data support the hypothesis that SHP-1 negatively regulates IL-6-driven Th17 development via limiting STAT3 activation.

**SHP-1 negatively regulates IL-21 mediated signaling in a T cell intrinsic manner.**

IL-21 has been identified as a second cytokine that can induce STAT3 activation and thereby mediate Th17 cell differentiation along with TGF-\( \beta \). Therefore, we next addressed whether SHP-1 also regulates IL-21-driven Th17 differentiation. We performed \textit{in vitro} Th17 differentiation assays using purified SHP-1-deficient and wild type T cells in response to a range of IL-21 concentrations under conditions of optimal TGF-\( \beta \) and TCR stimulation. \textit{me}+/ T cells develop increased percentages and absolute numbers of Th17 cells in response to IL-21 compared to +/+ T cells (Fig. 7 A-C). Furthermore, \textit{me}+/ T cells have a \( \sim \)2-fold lower EC\(_{50} \) than +/+ T cells (5 ng/ml IL-21 vs. 10 ng/ml IL-21), indicating that SHP-1 negatively regulates IL-21-mediated signaling and IL-21-driven Th17 differentiation. In contrast to what was observed in response to IL-6, \textit{me}+/ cells continue to hyper-respond even at higher IL-21 concentrations. Surface IL-21R levels, and MFI values are comparable between +/+ and \textit{me}+/ splenic T cells, (Fig. 7D) confirming that SHP-1 affects IL-21-mediated signaling downstream of the IL-21R, and that SHP-1-
deficient T cells have an intrinsic ability to hyper-respond to IL-21 stimulation. Collectively, these data demonstrate that SHP-1 negatively regulates IL-6- as well as IL-21-driven Th17 development.

**SHP-1 regulates STAT3 phosphorylation in response to IL-21 stimulation.**

Since SHP-1-deficient T cells hyper-respond to IL-21-driven Th17 differentiation, we asked whether IL-21-mediated STAT3 signaling is also regulated by SHP-1. Purified CD4+ T cells from +/+ and mel/+ mice were stimulated with IL-21 and the levels of induced phosphorylated STAT3 were measured. mel/+ T cells showed overall increased levels of phosphorylated STAT3 compared to +/+ T cells, but unchanged kinetics of phosphorylation (Fig. 7 E-F, left panel). When mel/+ mice that expressed STAT3 protein levels comparable to +/+ mice were analyzed, a considerable increase in relative STAT3 phosphorylation was observed (Fig. 7 E-F, right panel). This correlated well with the significant difference in IL-21-driven Th17 development between mel/+ and +/+ T cells (Fig. 7 A-C). These data indicate that SHP-1 regulates STAT3 activity by negatively regulating STAT3 phosphorylation in response to IL-21 stimulation. Collectively, our data support a model where SHP-1 limits IL-6- and IL-21-driven Th17 development, via decreasing STAT3 activation.
Discussion

To date, numerous studies have identified transcription factors that are critical for Th17 differentiation, but the upstream signaling events that modulate these transcription factors are less well understood. STAT3 is one of the key transcription factors known to promote Th17 differentiation. Here, we identify SHP-1 as a critical negative regulator of STAT3 signaling and IL-6 and IL-21-driven Th17 cell development. Our data suggest that SHP-1 can regulate Th17 differentiation via two mechanisms; first by negatively regulating STAT3 phosphorylation in response to IL-6 and IL-21 stimulation and second by limiting the levels of STAT3 protein available for activation. Since we observed that there are no differences in stat3 transcription between wild type and SHP-1 deficient T cells, these data suggest that SHP-1 limits STAT3 at the protein level, perhaps by indirectly regulating its degradation.

Our in vitro Th17 skewing studies identified SHP-1 as a negative regulator of both IL-6 and IL-21-driven Th17 development. To address whether SHP-1 might also be enhancing Th17 cell survival or expansion, we assessed these parameters in Th17 cultures and observed comparable levels of cell survival and proliferation between +/+ and mel/+ T cells (data not shown). This finding is consistent with our hypothesis that SHP-1 regulates Th17 differentiation and that differences in Th17 differentiation potential between +/+ and mel/+ T cells are not due to selective expansion or survival of the SHP-1-deficient T cells.

Recent studies have demonstrated that the hypoxia-inducible factor 1 (HIF-1α) is induced by STAT3 and promotes Th17 development through direct transcriptional activation of RORγt, an essential transcription factor for Th17 differentiation. Therefore, we tested whether SHP-1 affects the generation of HIF-1α at the transcriptional level. qRT-PCR for HIF-1α mRNA indicated that HIF-1α induction was comparable
between Th17 skewed +/+ and me+/+ cells, suggesting that this pathway is not being affected by SHP-1 deficiency (Fig. S2 A-B). Additionally, we examined the mRNA levels of Th17 family cytokines il17a, il17f, il22, and the lineage-specific transcription factor RORγt in Th17 skewed +/+ and me+/+ cells by qRT-PCR. While il-22 and Rorc expression levels were comparable between Th17 skewed +/+ and me+/+ cells, il17a and il17f induction was elevated in Th17 skewed me+/+ cells consistent with an upregulation of Th17 cytokine expression in SHP-1 deficient cells (Fig. S2 A-B).

Colitis is believed to be a Th17-mediated disease. Despite the increase in resident colonic Th17 cells, we did not detect any signs of colitis in any of the SHP-1-deficient mice. While there are several potential explanations for the lack of any pathogenesis, the following is perhaps the most likely one. Recent studies suggest that “non-pathogenic” Th17 cells can develop under specific cytokine and TCR stimuli. While the precise components of “non-pathogenic” Th17 development are not fully defined, lowered SHP-1 levels could promote the generation of non-pathogenic Th17 cells in the intestines of these mice.

SHP-1-deficient cells are capable of being pathogenic and inducing colitis mediated through T cell transfer. SHP-1-deficient and wild type T cells were equally pathogenic, and both cell types generated comparable Th17 populations, upon transfer. This suggests that in this model using a lymphopenic host, different factors such as homeostatic T cell expansion, may be the main force in driving T cell proliferation and differentiation, thereby overriding any differences in the response to the Th17-promoting cytokine milieu found in a non-lymphopenic mouse. To assess, whether SHP-1 deficiency affects the kinetics of colitis development, we also examined mice at earlier time points and found no differences indicating that SHP-1 does not influence the timing of Th17 development (data not shown). At earlier times points before the appearance of colitis or
Th17 T cells (4-6 weeks following T cells transfer), we observed the development of CD4^IFNγ^ T cells populations, which has been described for other autoimmune models, such as EAE and orchitis \textsuperscript{43,44}. However, these populations were comparable between recipients of +/+ or me/+ T cells, consistent with what we had observed \textit{in vitro}.

SSG-treatment of T cells results in hyper-responsiveness to IL-6 with increased \textit{in vitro} Th17 development. Interestingly, SSG is currently being tested in phase I and II clinical trials as a possible treatment option for patients with advanced solid tumors, as well as hematopoietic malignancies such as lymphomas, or myelomas \textsuperscript{25-27}. Current studies suggest that the presence of Th17 cells can be either beneficial or detrimental for the cancer patient, depending on the type of cancer \textsuperscript{13,14,16,45}. Therefore, our findings that SHP-1 deficiency/SSG treatment promotes IL-6 and IL-21-driven Th17 development, could be of high clinical significance, and suggest SHP-1 as a potential new therapeutic target for manipulating Th17 differentiation \textit{in vivo}. 
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Authorship Contributions

I.S.M. designed the research, performed the experiments, analyzed the results, created the figures, and wrote the manuscript.
K.S.T. analyzed histological slides and scored pathology of colitis experiments.
U.M.L. designed the research, supervised planning of experiments, helped with data analysis, figure preparation, and manuscript writing.

Disclosure of Conflicts

The authors declare no competing financial interests.
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Figure Legends

Figure 1.

SHP-1 limits natural intestinal Th17 development.

A. IL-17 expression of CD4+ gated T cell population isolated from the Peyer’s patches of +/+, me/+ and me/me BALB/c DO11.10 and C57BL/6 mice. +/+ and me/+ mice have comparable numbers of CD4+ cells, while me/me mice have 50% reduced CD4+ cell numbers in their Peyer’s patches correlating with their decreased size. B. Representative colon segments from +/+ (n = 8), me/+ (n = 7), and me/me (n = 8) BALB/c DO11.10 mice (H&E stain x200 magnification). C-D. Percentages of IL17A/F+ cells within the CD4+ compartment isolated from the Peyer’s patches (C) and intestinal lamina propria (D) of mice with the indicated genotypes. E. IL-17 expression of CD4+ gated T cell population isolated from inguinal lymph nodes, mesenteric lymph nodes, and spleen of +/+, me/+ and me/me C57BL/6 and BALB/c DO11.10 mice. The presented data are representative of 2-4 independent experiments with multiple mice for each genotype per experiment.

Figure 2.

SHP-1-deficient T cells are pathogenic and able to induce colitis.

CD4+CD25− CD45RBhi T cells (5x10^5) isolated from +/+ or me/+ mice were transferred into rag1-/- recipients. A. Representative colon segments of recipient mice 7-8 weeks after adoptive T cell transfer (H&E, +/+, me/+ and PBS, x200). The arrow points to a giant cell among inflammatory cells. B. Pathological scoring of colon segments from recipient mice 7-8 weeks after adoptive T cell transfer (+/+ v.s me/+ p value = 0.5 NS; +/+ v.s PBS p value = 0.001; me/+ vs. PBS p value = 0.02). C-D. Numbers of IL17A/F+ T cells within the CD4+ compartment isolated from the lamina propria (C, +/+ v.s me/+ p value =0.5 NS) and Peyer’s patches (D, +/+ v.s me/+ p value = 0.7 NS) of the indicated donor derived
populations 7-8 weeks after transfer. All data presented here are representative of 3 independent experiments with multiple mice for each genotype per experiment.

**Figure 3.**

**SHP-1 negatively regulates IL-6 mediated Th17 development.**

**A.** Representative IL-6 dose response assay. CD4+ T cells from +/+ and me+/ C57BL/6 mice that were cultured under Th17-inducing with the indicated IL-6 concentrations followed by intracellular staining for IFNγ and IL-17. Profiles show CD4+-gated population.

**B.** Relative Th17 T cell generation from IL-6 dose response assays presented in A. Percentages of maximal Th17 cells generated in cultures (max % Th17 cells: 37% +/+,
40% me/+ ) were set to 100% to determine EC50. EC50 were calculated as: +/+ cells 1.0 ng/ml IL-6 (n = 4), me/+ cells 0.25 ng/ml IL-6 (n = 7); p value = 0.001. **C.** IL6R surface expression of splenic +/+ , me/+ or me/me CD4+ T cells. The presented data are representative of 2-3 independent experiments with multiple mice for each genotype per experiment.

**Figure 4.**

**SHP-1 negatively regulates IL-6 mediated Th17 development in a T cell intrinsic manner.**

**A.** Graphic Map of the construct used to generate DN-SHP-1 mice. **B.** Representative IL-6 dose response assay of DN-SHP-1 expressing and non-expressing T cells that were cultured under Th17-inducing, with the indicated IL-6 concentrations, followed by intracellular staining for IFNγ and IL-17. Profiles show CD4+-gated population. **C.** Relative Th17 T cell generation from IL-6 dose response assays as described in B. (max % Th17 cells: 35% non-expressers, 34% DN-SHP-1-expressers). EC50: DN-SHP-1-expressers 1.0
ng/ml IL-6 (n = 3), non-expressers 2.5 ng/ml IL-6 (n = 4); p value = 0.13. Data are representative of 2 independent experiments with multiple mice for each genotype.

Figure 5.

SSG-mediated SHP-1 inhibition increases Th17 development.

A. Relative Th17 T cell generation in IL-6 dose response assay comparing SSG-treated and un-treated +/+ T cells. (max % Th17 cells: 46% untreated, 47% SSG treated). EC$_{50}$: untreated +/+ cells 1.0 ng/ml IL-6 (n = 2), SSG-treated cells 0.25 ng/ml IL-6 (n = 2); p value = 0.04. B. IL17A/F expression of CD4$^+$ T cells from +/+, me/+ and me/me BALB/c DO11.10 mice that were cultured with or without SSG under Th17-inducing conditions with the indicated concentrations of IL-6. Profiles show CD4$^+$-gated population. Data are representative of 2 independent experiments with multiple mice for each genotype.

Figure 6.

SHP-1 negatively regulates IL-6-induced STAT3 activation.

A. CD4$^+$ T cells (0.7x10$^6$) purified from +/+, me/+ and me/me C57BL/6 mice were stimulated with IL-6 for the indicated times. Cells were assessed for STAT3 phosphorylation (Tyr705), STAT3, and β-actin protein expression by immunoblotting. Numbers represent relative band densities in arbitrary units. B. Kinetics of IL-6 induced STAT3 phosphorylation presented in A. pSTAT3 band densities normalized to β-Actin (left panel) and pSTAT3 band densities normalized to STAT3 (right panel). C. CD4$^+$ T cells (0.7x10$^6$) purified from +/- and me/+ mice were stimulated with IL-6 for 10 min. and analyzed as described in A. D. qRT-PCR analysis of STAT3 mRNA isolated from splenic CD4$^+$ T cells of +/-, me/+ and me/me C57BL/6 mice. STAT3 mRNA expression levels
were normalized to *HPRT1* and levels for +/+ set to 1. All data presented here are representative of 2-3 independent experiments with multiple mice for each genotype.

**Figure 7.**

**SHP-1 negatively regulates IL-21-mediated Th17 development.**

**A.** Representative IL-21 dose response assay. CD4⁺ T cells from +/+ and *me/+* C57BL/6 mice that were cultured under Th17-inducing with the indicated IL-21 concentrations followed by intracellular staining for IL-17A/F. Profiles show CD4⁺-gated population. **B.** Relative Th17 T cell generation in IL-21 dose response assays comparing +/+ and *me/+* T cells. (max % Th17 cells: 23% +/+, 30% *me/+*). **EC₅₀:** +/+ cells 10 ng/ml IL-21 (n = 6), *me/+* cells 5 ng/ml IL-21 (n = 6); *p* value = 0.01. **C.** Representative graph of absolute numbers of IL17⁺CD4⁺ T cells generated in IL-21 dose response assays, as depicted in A. **D.** IL21R surface expression of splenic CD4⁺ T cells. **E.** CD4⁺ T cells (0.7x10⁶) purified from +/+, *me/+* and *me/me* C57BL/6 mice were stimulated with IL-21 for the indicated times and assessed for STAT3 phosphorylation (Tyr705), STAT3, and β-actin protein expression by immunoblotting. Numbers represent relative band densities in arbitrary units. **F.** Kinetics of IL-21 induced STAT3 phosphorylation presented in E. pSTAT3 band densities normalized to β-Actin (left panel) and pSTAT3 band densities normalized to STAT3 (right panel). All data represent 2-3 independent experiments with multiple mice per genotype.
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The tyrosine phosphatase SHP 1 as a new player that dampens murine Th17 development

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